GFP-FRNK Disrupts Focal Adhesions and Induces Anoikis in Neonatal Rat Ventricular Myocytes

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Abstract—Focal adhesion kinase (FAK) is a nonreceptor protein tyrosine kinase involved in adhesion-dependent signal transduction. FAK is highly expressed in cultured neonatal rat ventricular myocytes (NRVMs) and undergoes tyrosine autophosphorylation in response to cell adhesion, stretch, and growth factor stimulation. We previously showed that inhibition of FAK phosphorylation by adenovirally mediated overexpression of FRNK (the autonomously expressed C-terminal domain of FAK) prevented endothelin-1 (ET)-induced NRVM hypertrophy. One question raised by these studies was whether FRNK localized to focal adhesions and displaced FAK from sites required for downstream signaling. Therefore, we constructed a replication-defective adenosine encoding a GFP-FRNK fusion protein (Adv-GFP-FRNK) and examined its effects on NRVM cytoarchitecture and signaling. Uninfected NRVMs contained small amounts of endogenous FRNK. NRVMs infected with Adv-GFP-FRNK expressed much larger amounts of a 66-/68-kDa protein that localized to costameres and focal adhesions. GFP-FRNK overexpression suppressed basal and ET-induced FAK phosphorylation and also inhibited ET-induced phosphorylation of PYK2, the other member of the FAK family of nonreceptor protein tyrosine kinases. In contrast, GFP-FRNK overexpression did not prevent ET-induced ERK, JNK, or p70S6K phosphorylation. Furthermore, GFP-FRNK resulted in the loss of detectable FAK and paxillin in focal adhesions, which was accompanied by reduced levels of total paxillin and, ultimately, cell detachment and apoptosis. We conclude that FRNK functions as a dominant-negative inhibitor of adhesion-dependent signaling by displacing FAK from focal adhesions and interfering with the anchorage of NRVMs that is necessary for cell survival, a process known as anoikis. (Circ Res. 2002;90:1282-1289.)

Key Words: focal adhesion kinase □ apoptosis □ adenovirus □ PYK2 □ paxillin

Costameres are band-like cytoskeletal structures that are adjacent to the sarcolemmal membrane surrounding individual Z-discs that couple myofibrils to the extracellular matrix (ECM). This important structural site is linked to the ECM through integrins in the sarcolemma. Terracio et al.1 first demonstrated the presence of β1-integrins at the costamere surface of freshly isolated adult cardiac muscle cells. Costamere-like structures containing integrins are found in cultured neonatal and adult cardiac myocytes.2 Cultured cardiomyocytes also form focal adhesions (similar to focal adhesions and displaced FAK from specific binding sites required for downstream signaling. Therefore, we constructed
a replication-defective adenovirus encoding a green fluorescent protein (GFP-FRNK) fusion protein (Adv-GFP-FRNK) to examine the effect of FRNK on NRVM cytoarchitecture and signaling. In this study, we demonstrate that FRNK is endogenously expressed in NRVMs and describe a potential mechanism whereby FRNK inhibits both basal and ET-induced FAK autophosphorylation. We also describe the functional consequence of GFP-FRNK overexpression in NRVMs.

Materials and Methods

Reagents

The reagents used are listed in the online data supplement, which is available at http://www.circresaha.org

Cell Culture

Animals used in these experiments were handled in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Council of the American Physiological Society. Ventricular myocytes were isolated from the hearts of 2-day-old Sprague-Dawley rats (Zivic Laboratories, Inc., Zelienople, Pa) by collagenase digestion, as previously described. Myocytes were preplated for 1 hour in serum-free PC-1 medium to reduce nonmyocyte contamination. The nonadherent NRVMs were then plated at a density of 1600 cells per mm² onto collagen-coated chamber slides or 35-mm dishes and left undisturbed in a 5% CO² incubator for 14 to 18 hours. Unattached cells were removed by aspiration, washed twice in HBSS, and the attached cells were maintained in a solution of DMEM/Medium 199 (4:1) containing antibiotic/antimycotic solution. Cardiomyocytes were infected (60 minutes, 25 °C) with gentle agitation with replication-defective adenoviruses diluted in DMEM/Medium 199. Medium was then replaced with virus-free DMEM/Medium 199, and the cells cultured for an additional 48 to 72 hours.

Adenoviral Constructs

A replication-defective adenovirus encoding chick FRNK (Adv-FRNK) was constructed as previously described. An adenovirus encoding a GFP-FRNK fusion protein (Adv-GFP-FRNK) was engineered by cloning chick FRNK cDNA (kindly provided by J.T. Parsons, University of Virginia, Charlottesville, Va) into the EcoRI site of pEFGP-C1 vector (Clontech). The GFP-FRNK insert was then subcloned into the pShuttle-CMV plasmid (Pmel site) by in vitro blunt ligation. The pShuttle-CMV expression cassette containing GFP-FRNK was excised by restriction enzyme digestion, subcloned into pAdeno-X viral DNA (Clontech), and linearized according to manufacturer’s instructions. The linearized pAdeno-X+GFP-FRNK was introduced into HEK293 cells using a liposome-based transient transfection procedure (SuperFect, Qiagen). GFP-FRNK adenovirus (Adv-GFP-FRNK) was amplified from cell extracts and purified by CsCl gradient centrifugation. The multiplicity of viral infection (moi) was determined by viral dilution assay in HEK293 cells grown in 96-well clusters. An adenovirus expressing GFP alone (Adv-GFP) was used to control for nonspecific effects of adenoviral infection.

Immunolocalization

NRVMs grown on chamber slides were fixed and permeabilized as previously described. Myocytes were stained with rhodamine phalloidin (to visualize actin filaments), FAK (with either a polyclonal antibody [pAb] directed to the COOH-terminus, which recognizes both FAK and FRNK, or a monoclonal antibody [mAb] directed to the kinase domain, which recognized only endogenous FAK), or paxillin. Appropriate FITC- or rhodamine-conjugated secondary antibodies were used to visualize the proteins of interest. Fluorescently labeled cells were then viewed using a Zeiss model LSM 510 laser scanning confocal microscope.

Immunoprecipitation and Western Blotting

NRVMs were washed once in ice-cold PBS and homogenized in a lysis buffer containing 1% Triton X-100 and 0.1% SDS for Western blotting experiments. For immunoprecipitation experiments, NRVMs were washed once in ice-cold PBS and scraped in homogenized buffer (0.5% Nonidet P-40, 25 mmol/L HEPES [pH 7.4], 150 mmol/L NaCl, 1.5 mmol/L MgCl², 1 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 10 mmol/L NaF, 0.1 mmol/L sodium orthovanadate, and 1% sodium deoxycholate). Equal amounts of extracted cellular proteins were immunoprecipitated with either nonimmune IgG, anti-FAK pAb, or anti-paxillin mAb overnight at 4°C followed by the addition of protein A or protein G beads. For both immunoprecipitation and Western blotting experiments, equal amounts of extracted cellular proteins were separated on 7.5% SDS-polyacrylamide gels with 4% stacking gels. Proteins were then transferred to PVDF membranes using the recommended transfer buffer. Western blots were probed with antibodies specific for GFP, FAK, paxillin, or the phosphorlated forms of FAK at Y397, ERK1/2, JNK1/2, and P70S6K. Primary antibody binding was detected with horseradish peroxidase–conjugated goat anti-mouse or goat anti-rabbit secondary antibody, where appropriate, and visualized by enhanced chemiluminescence (Amersham). Band intensity was quantified using laser densitometry.

Subcellular Fractionation

NRVMs were scraped in homogenization buffer (2 mmol/L EDTA, 2 mmol/L EGTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 500 μmol/L sodium orthovanadate, 1 mmol/L PefaBloc, and 200 mmol/L Tris-HCl [pH 7.5]), sonicated, and centrifuged (100 000 g, 1 hour). The supernatant (S) fraction was lyophilized to dryness. The particulate (P) fraction was resuspended in a buffer containing 1% Triton X-100 and 0.1% SDS, sonicated, and recentrifuged (10 000 g, 20 minutes). The S and P fractions were then resuspended in equal volumes of lysis buffer, and equal volumes from each fraction were separated by SDS-PAGE and Western blotting.

Detection of Apoptosis

DNA fragmentation was assayed as previously described. Fragmented DNA was separated from intact high-molecular-weight genomic DNA, and the fragments were resolved by agarose gel electrophoresis.

Data Analysis

Results were expressed as mean±SEM. Normality was assessed using the Kolmogorov-Smirnov test, and homogeneity of variance was assessed using Levene’s test. Data were compared by 1-way blocked ANOVA, followed by the Student-Newman-Keuls test. Differences among means were considered significant at P<0.05. Data were analyzed using the SigmaStat Statistical Software Package, version 1.0 (Jandel Scientific, San Rafael, Calif).

Results

Detection of Endogenous FRNK and GFP-FRNK in NRVMs

As seen in Figure 1A, a pAb antibody specific for the C-terminal portion of FAK cross-reacted with several proteins in uninfected NRVM extracts. The major protein band (125 kDa) also cross-reacted with an N-terminal anti-FAK mAb, as well as a pAb specific for FAK phosphorlated at Y397 (data not shown). Of particular interest, a 41-/43-kDa doublet was also detected when large amounts (500 μg) of extracted NRVM proteins were examined. This doublet comigrated with proteins expressed in NRVMs infected with Adv-FRNK. However, the doublet did not cross-react with either the N-terminal or pFAK antibodies (data not shown),
indicating that the bands were endogenous FRNK or, less likely, C-terminal degradation products of endogenous FAK.

Experiments were then performed to demonstrate the cross-reactivity of GFP-FRNK with anti-FAK and anti-GFP antibodies in NRVMs infected with Adv-GFP-FRNK. As seen in Figure 1B, the C-terminal anti-FAK antibody cross-reacted with endogenous FAK and with GFP-FRK (66/68 kDa) (left blot). No endogenous FRNK was detected in this experiment, owing to the small amount of NRVM protein extract examined and the brief exposure time of the film. An anti-GFP antibody cross-reacted with GFP-FRNK and also with GFP (27 to 29 kDa) expressed by the control adenovirus (right blot).

**GFP-FRNK Localizes to Costameres and Focal Adhesions**

Adv-GFP– and Adv-GFP-FRNK–infected NRVMs were examined by confocal microscopy 48 hours after infection. Cells were counterstained with rhodamine phalloidin to visualize actin filaments (Figure 2). Infected cells were distinguished from adjacent uninfected NRVMs by the presence of intracellular green fluorescence. Whereas GFP was diffusely distributed throughout the cytoplasm, GFP-FRNK was distributed in a striated pattern consistent with that of costameres and at the ends of actin filaments, where focal adhesions are present.

**Effects of GFP-FRNK Overexpression on ET-Induced Signal Transduction**

To determine whether GFP-FRNK inhibited FAK-dependent signal transduction, NRVMs were infected with Adv-GFP or Adv-GFP-FRNK (48 hours) and then stimulated with ET (10 minutes). A representative Western blot is shown in Figure 3A, and the results of 6 experiments are summarized in Figure 3B. As is evident from the figure, NRVMs contain substantial amounts of FAK phosphorylated at Y397. ET

**Figure 1.** Detection of endogenous FRNK and GFP-FRNK in NRVMs. NRVMs were maintained in control medium (Uninfected) or infected with Adv-FRNK for use as a positive control for FRNK (24 hours, 25 moi) (A). Western blots (500 μg of extracted protein from uninfected cells; 200 μg from Adv-FRNK–infected cells) were probed with anti-FAK pAb, which is specific for the C-terminal portion of FAK as well as FRNK. B, NRVMs were maintained in control medium (Uninfected) or infected with Adv-GFP or Adv-GFP-FRNK (48 hours; 10 moi). Western blots (50 μg of extracted protein) were probed with either anti-FAK pAb (left) or anti-GFP mAb (right).

**Figure 2.** GFP-FRNK localizes to costameres and focal adhesions. NRVMs were infected (48 hours, 10 moi) with Adv-GFP (A) or Adv-GFP-FRNK (B). Cells were fixed, labeled with rhodamine-conjugated phalloidin, and viewed under a laser confocal microscope (1-μm optical sections obtained at the cell-substratum interface). Actin filaments (red) were apparent in both Adv-GFP– and Adv-GFP-FRNK–infected cells. The localization of GFP appeared mainly cytoplasmic, whereas GFP-FRNK was distributed in a striated pattern and at the ends of actin filaments (green). Areas of colocalization appear as yellow.
stimulation resulted in a further 2-fold increase in pFAK Y397 compared with unstimulated Adv-GFP–infected cells. In contrast, GFP-FRNK overexpression markedly reduced both basal and ET-induced FAK phosphorylation. Although ET increased endogenous pFAK Y397 levels in GFP-FRNK–expressing cells, this increase did not reach statistical significance (Figure 3B). The reductions in basal and ET-induced FAK phosphorylation were not due to a reduction in the total amount of FAK in GFP-FRNK–expressing cells, because endogenous FAK levels were similar in both GFP- and GFP-FRNK–overexpressing NRVMs (Figures 3C and 3D).

We then examined the effects of GFP-FRNK overexpression on other signal transduction pathways. Because FRNK overexpression has been reported to interfere with PYK2 activation in other cell types, we examined the effect of GFP-FRNK overexpression on basal and ET-induced PYK2 phosphorylation. As seen in Figure 3E (top blot), ET-induced PYK2 Y402 phosphorylation was markedly suppressed in Adv-GFP-FRNK–infected NRVMs. However, GFP-FRNK overexpression did not substantially interfere with either basal or ET-induced activation of ERK1/2, JNK1/2, or p70 S6 kinase (Figure 3E).

**GFP-FRNK Overexpression Results in the Loss of FAK- and Paxillin-Positive Focal Adhesions**

One explanation for the inhibition of FAK phosphorylation in GFP-FRNK–overexpressing cells is that FAK was displaced from focal adhesions but remained in a nonfunctioning intracellular pool. Therefore, we examined the colocalization of FAK and paxillin, a known FAK substrate and focal adhesion component. As seen in Figure 4, double-label confocal microscopy revealed that paxillin (Figure 4A) and FAK (Figure 4B) were colocalized (Figure 4C) at the cell-substratum interface. Although FAK was present in the same focal adhesion sites as paxillin, additional FAK staining was detected in the perinuclear region that was often devoid of paxillin staining. Next, Adv-GFP-FRNK–infected cells were labeled with a mAb specific for the kinase domain of FAK. This antibody recognizes endogenous FRNK but not GFP-FRNK. As seen in Figure 5A, endogenous FAK was localized to focal adhesions in NRVMs that did not express GFP-FRNK. However, GFP-FRNK was detected at the cell-substrate interface consistent with the appearance of focal adhesions, but the endogenous FAK appeared mainly perinuclear. The lack of colocalization of the two proteins provides evidence for the displacement of endogenous FAK from focal adhesions by GFP-FRNK overexpression. In Figure 5B, paxillin localized to focal adhesions in myocytes that did not express GFP-FRNK. However, in NRVMs overexpressing GFP-FRNK, there was an overall loss of paxillin-positive focal adhesions. The remaining paxillin appeared to be distributed diffusely throughout the cell.

**GFP-FRNK Coimmunoprecipitates With Paxillin**

As further evidence for the displacement of FAK from focal adhesions by GFP-FRNK, coimmunoprecipitation experiments were performed. Western blots (50 μg of extracted protein) were probed with either a pAb antibody specific for the FAK Y397 autophosphorylation site (A) or a pAb that recognizes both unphosphorylated and phosphorylated forms of endogenous FAK as well as GFP-FRNK (C). B and D, Quantitative analysis of 6 Western blotting experiments. Levels of pFAK Y397 (B) and endogenous FAK (D) are shown, normalized to those observed after Adv-GFP infection (without ET treatment). E, Representative Western blots (50 to 100 μg of extracted protein) probed with pAbs specific for either the PYK2 Y402 phosphorylation site, the phosphorylated forms (p) of ERK1/2, JNK1/2, or P70S6K (E). The position of molecular weight markers is indicated to the left or right of each blot. Data are mean ± SEM. **P < 0.05 vs untreated Adv-GFP; *P < 0.05 vs its corresponding untreated control.

**Figure 3. Effects of GFP-FRNK overexpression on ET-induced signal transduction.** NRVMs were infected (10 moi, 48 hours) with Adv-GFP or Adv-GFP-FRNK and then treated with ET (10 nmol/L, 10 minutes). Western blots (50 μg of extracted protein) were probed with either a pAb antibody specific for the FAK Y397 autophosphorylation site (A) or a pAb that recognizes both unphosphorylated and phosphorylated forms of endogenous FAK as well as GFP-FRNK (C). B and D, Quantitative analysis of 6 Western blotting experiments. Levels of pFAK Y397 (B) and endogenous FAK (D) are shown, normalized to those observed after Adv-GFP infection (without ET treatment). E, Representative Western blots (50 to 100 μg of extracted protein) probed with pAbs specific for either the PYK2 Y402 phosphorylation site, the phosphorylated forms (p) of ERK1/2, JNK1/2, or P70S6K (E). The position of molecular weight markers is indicated to the left or right of each blot. Data are mean ± SEM. **P < 0.05 vs untreated Adv-GFP; *P < 0.05 vs its corresponding untreated control.

**Figure 4. FAK and paxillin colocalize to focal adhesions.** NRVMs were maintained in culture medium, then fixed and double-labeled with an anti-α-paxillin mAb (A) and an anti-FAK pAb (B). Rhodamine-conjugated goat anti-mouse IgG (red) and FITC-conjugated goat anti-rabbit IgG (green) were used for visualization by laser confocal microscopy. (1-μm optical sections obtained at the cell-substratum interface). Areas of colocalization appear as yellow (C).
ments were performed to examine whether FAK binds to paxillin in NRVMs and also whether paxillin physically interacts with GFP-FRNK. Figure 5C shows a representative experiment in which NRVM extracts (prepared in the absence of Triton X-100 and SDS) were immunoprecipitated with C-terminal anti-FAK pAb, and the resulting immunoprecipitates probed with either anti-paxillin or anti-FAK antibodies. A small amount of paxillin was found to coimmunoprecipitate with endogenous FAK in control cells expressing GFP (left panel). However, in cells overexpressing GFP-FRNK, much larger amounts of paxillin coimmunoprecipitated with GFP-FRNK rather than with endogenous FAK in Adv-GFP-FRNK–infected NRVMs. Coimmunoprecipitation experiments with anti-paxillin antibody yielded similar results (data not shown). Thus, these data provide additional evidence for the displacement of FAK by GFP-FRNK from NRVM focal adhesions.

GFP-FRNK Overexpression Results in the Loss of Paxillin

As was seen in Figure 5B, GFP-FRNK overexpression appeared to reduce the total amount of paxillin staining in infected cells. We therefore examined the total levels of paxillin in uninfected, Adv-GFP–infected, and Adv-GFP-FRNK–infected NRVMs by Western blotting. As seen in Figures 6A and 6B, there was no significant difference in the amount of paxillin in cell extracts of uninfected and Adv-GFP–infected NRVMs. However, paxillin levels were reduced to 24 ± 6% of control levels in Adv-GFP-FRNK–infected cells. We next examined the subcellular distribution of paxillin in Adv-GFP– and Adv-GFP-FRNK–infected NRVMs. Surprisingly, 42 ± 8% of the total amount of paxillin was present in the S fraction of GFP-overexpressing cells (Figures 6C and 6D), indicating that unlike FAK, there is a large pool of paxillin in the cytoplasm of NRVMs. GFP-FRNK overexpression resulted in a marked reduction in paxillin from both subcellular fractions.

Long-Term Overexpression of GFP-FRNK Results in Cell Death by Apoptosis not Necrosis

As seen in Figure 7C, long-term (72 hours) overexpression of GFP-FRNK resulted in cell rounding and detachment from

Figure 5. GFP-FRNK overexpression redistributes FAK and paxillin. NRVMs were infected (10 moi; 24 to 48 hours) with Adv-GFP-FRNK. Cells were fixed and labeled with either a mAb specific for the FAK kinase domain (which cross-reacts with endogenous FAK but not GFP-FRNK) (A) or an anti-α-paxillin antibody (B). Endogenous FAK and paxillin were visualized with a rhodamine-conjugated goat anti-mouse IgG and visualized by laser confocal microscopy. Adv-GFP-FRNK–infected cells (green) are distinguished from adjacent noninfected cells by the absence of a green fluorescence signal. Any areas of colocalization appear as yellow. C, NRVMs were maintained in control medium (Uninfected) or infected with Adv-GFP or Adv-GFP-FRNK (48 hours; 10 moi). Equal amounts of extracted cellular protein (800 μg) were immunoprecipitated with either nonimmune IgG (mock) or Anti-FAK pAb and separated by SDS-PAGE. Immunoblots were probed with anti-paxillin mAb, then stripped and reprobed with anti-FAK pAb. The position of the molecular weight markers is indicated to the right of each blot.

Figure 6. Overexpression of GFP-FRNK results in the loss of paxillin. NRVMs were maintained in control medium (Uninfected) or infected (10 moi; 48 hours) with Adv-GFP or Adv-GFP-FRNK. Western blots of whole cell lysates (50 μg each of total cellular protein) were probed with anti-paxillin mAb (A). C, cells were lysed in the absence of detergents and subjected to differential centrifugation to generate soluble (S) and particulate (P) fractions. Equal volumes of each fraction were subjected to Western blotting. Blots were probed with anti-paxillin mAb. B and D, Quantitative analysis of 3 to 5 Western blotting experiments. Data are mean ± SEM. *P < 0.05 vs its corresponding Adv-GFP fraction.
by Hoffman modulation contrast (left) or GFP with Adv-GFP (B) or Adv-GFP-FRNK (C). Cells were visualized in control conditions (Uninfected; A) or infected (10 moi; 72 hours) rounding and detachment. NRVMs were maintained under control (Figures 8E and 8F), NRVMs were treated with CaCl$_2$ (10 mmol/L, 6 hours) to induce calcium overload and cell necrosis, which was readily detected in the majority of myocytes as the inability to exclude propidium iodide from the nucleus. Finally, NRVMs were infected (72 hours) with Adv-GFP or Adv-GFP-FRNK and then analyzed for DNA fragmentation (Figure 8G). Cells overexpressing GFP-FRNK exhibited the characteristic degradation of DNA into oligonucleosomal-length fragments, indicating that the functional consequence of overexpressing GFP-FRNK was cell adhesion-dependent apoptosis, or anoikis.

**Discussion**

In this report, we provide evidence that GFP-FRNK localizes to focal adhesions where it interferes with adhesion-dependent cell signaling. Interruption of FAK autophosphorylation resulted in disruption of focal adhesion architecture, ultimately leading to anoikis. These novel results may help clarify the importance of FAK in providing cell survival signals in NRVMs and may also provide some insight into the mechanism whereby endogenously expressed FRNK regulates cardiomyocyte adhesion, growth, and survival.

We have previously demonstrated that FAK is a critical component of the signaling pathways that are necessary for cellular hypertrophy induced by Gq protein–coupled receptor agonists in cardiomyocytes$^{20}$ and in vascular smooth muscle cells.$^{20}$ In NRVMs, ET-induced FAK and paxillin phosphorylation were both acutely inhibited in cells infected with a replication-defective adenovirus encoding chick FRNK. Inhibition of FAK signaling subsequently resulted in the failure of NRVMs to synthesize contractile proteins$^5$ and to assemble them into sarcomeres.$^6$ Similarly, in cultured rat aortic smooth muscle cells, FRNK overexpression blocked angiotensin II–induced FAK tyrosine phosphorylation, markedly inhibited protein synthesis, and disrupted actin stress fiber assembly.$^{20}$

In the present study, we have used an Adv-GFP-FRNK adenoviral construct to identify the mechanism whereby FRNK inhibits FAK-dependent signal transduction. We found that FAK colocalized with the cytoskeletal adapter protein paxillin in regions of the cardiomyocyte consistent with the appearance of focal adhesions. GFP-FRNK colocalized to the same cytoskeletal structures. FRNK localization to focal adhesions has also been observed in chick embryo fibroblasts, which express high levels of the endogenously expressed protein.$^{22}$ Furthermore, we found that both basal and ET-induced pFAK Y397 were decreased after GFP-FRNK overexpression, but the total amount of FAK was not significantly affected. Similar results have been reported recently in GFP-FRNK–infected NRVMs in response to phenylephrine$^{14}$ and with a GFP fusion protein encoding only the C-terminal focal adhesion targeting (FAT) sequence of FAK.$^{23}$

Thus, the loss of phosphorylated FAK in GFP-FRNK–overexpressing NRVMs was not due to an overall reduction in FAK as a consequence of focal adhesion disruption and nonselective degradation of all of its components. Similarly, Richardson and Parsons$^{13}$ showed that FRNK overexpression in chicken embryonic cells inhibited the tyrosine phosphorylation of FAK, tensin, and paxillin. FRNK overexpression delayed focal adhesion formation, but vinculin-positive focal adhesions were still present and there was no decrease in total FAK, paxillin, or tensin levels. These results suggest that tyrosine phosphorylation of FAK is not required for the maintenance of focal adhesion structure in some cell types. Furthermore, embryonic cells derived from FAK null mice formed dense focal adhesions that demonstrated impaired cell spreading and migration.$^{24}$ We now show that in NRVMs overexpressing GFP-FRNK, FAK was displaced from focal adhesions, which was likely the result of

![Figure 7. Long-term overexpression of GFP-FRNK results in cell rounding and detachment. NRVMs were maintained under control conditions (Uninfected; A) or infected (10 moi; 72 hours) with Adv-GFP (B) or Adv-GFP-FRNK (C). Cells were visualized by Hoffman modulation contrast (left) or GFP fluorescent (right) microscopy (×20 objective for all fields).](http://circres.ahajournals.org/):
competitive binding of GFP-FRNK to paxillin and other cytoskeletal proteins via its identical C-terminal FAT sequence. Competitive binding of FAK and GFP-FRNK to paxillin was confirmed in coimmunoprecipitation experiments. However, endogenous FAK was not lost from NRVMs overexpressing GFP-FRNK. Preservation of FAK levels may be related to structural motifs within the N-terminal region of FAK. For instance, Schaller et al. showed that the N-terminal domain of FAK was capable of binding to peptides derived from \( \beta \)-integrin cytoplasmic domains. This interaction may stabilize FAK associations with integrins within focal adhesions. In addition, Girault et al. have indicated that the FAK N-terminal region bears substantial sequence homology to the filamentous actin capping proteins ezrin, radixin, and moesin. Thus, FAK still bound to both integrins and/or actin filaments might be expected to be retained in the cell. Nevertheless, further studies will be necessary to explore these possibilities.

In contrast to FAK, we found that GFP-FRNK overexpression caused the disappearance of paxillin from focal adhesion structures, as well as from the particulate fraction in subcellular fractionation experiments. In the case of this cytoskeletal protein, however, the total amount of paxillin was markedly reduced, perhaps by selective proteolytic degradation. Because paxillin is a known FAK substrate, GFP-FRNK overexpression in NRVMs must have inhibited FAK-dependent signaling, which was then accompanied by the loss of paxillin phosphorylation and subsequent disorganization of focal adhesions. Surprisingly, we also found a substantial amount of paxillin in the soluble fraction of control cardiomyocytes, and this pool of soluble paxillin also disappeared in response to GFP-FRNK overexpression. The function of the soluble paxillin pool is unknown but may reflect its role as an adapter protein for other soluble signaling kinases. For instance, PYK2, the other member of the FAK family of nonreceptor protein tyrosine kinases, is predominantly localized to the cytoplasm of both neonatal and adult cardiomyocytes. PYK2 also binds to paxillin, as well as other components involved in both adhesion-dependent and adhesion-independent signaling.

In this regard, we found that GFP-FRNK overexpression resulted in the loss of ET-induced PYK2 activation. These results suggest that PYK2 is downstream of agonist-induced FAK activation in NRVMs. Alternatively, transient localization of PYK2 in focal adhesions may be required for its activation in response to ET. Indeed, PYK2 contains a homologous FAT sequence, and overexpression of the C-terminal region of PYK2 markedly inhibited PYK2 but not FAK autoactivation in HEK293 cells. Nevertheless, it is conceivable that some of the effects of GFP-FRNK overexpression on NRVM cytoarchitecture and signaling may be in fact due to its inhibitory effects on PYK2-dependent signal transduction. Further studies are required to dissect this potential crosstalk in cardiomyocytes.

In contrast, GFP-FRNK overexpression did not substantially inhibit ET-induced ERK1/2, JNK1/2, or P70S6K activation. These data suggest that ET-induced activation of these protein kinases is not dependent on signaling through focal adhesions. Our results differ somewhat from those of Taylor et al., who demonstrated that GFP-FRNK inhibited ERK activation in response to phenylephrine. However, it should be pointed out that there are substantial differences in the culture conditions between the two studies, which may explain the differing results.

We demonstrated that the inhibition of basal pFAK Y397 by GFP-FRNK overexpression ultimately led to NRVM detachment and apoptosis. Cell adhesion-dependent apoptosis, or anoikis, has been shown in other cell types to be dependent on signals originating from FAK. For example, overexpression of a constitutively active form of FAK res-
cued suspended epithelial cells from anothai, and both the Y397 autophosphorylation site and K454, a site critical for kinase activity, were required for this effect.11 Thus, it seems that FAK tyrosine kinase activity is necessary to maintain cell survival. Nevertheless, it remains unknown whether the ability of FAK to suppress apoptosis is due to its role as a protein tyrosine kinase capable of directly phosphorylating downstream signaling kinases, adapter proteins, and cytoskeletal elements. On the other hand, FAK may indirectly prevent anoikis by providing a docking site for other signaling molecules (such as Src and phosphatidylinositol 3-kinase) that are clearly involved in cell survival signaling.11

FRNK is expressed as an autonomous protein in multiple tissues, including the embryonic, and, to a much lesser extent, in adult heart.12 In this report, we demonstrate that FRNK normally serves as a regulator of cellular processes involving FAK in cardiomyocytes, as well as in other cell types. Because FRNK consists of the identical 360 amino acids that comprise the C-terminal region of FAK,22 it therefore contains the same proline-rich regions and tyrosine phosphorylation sites that are involved in binding p130Cas, Grb2, GRAF, and paxillin. FRNK may thus act as an alternative substrate for Src family protein tyrosine kinases that bind to, and phosphorylate, FAK, thus competing with FAK for specific binding partners and diverting or extinguishing downstream signal transduction. However, our results indicate that GFP-FRNK overexpression also leads to the physical disruption of focal adhesion cytoarchitecture, which induces anoikis. Future studies are needed to clarify the structural versus signaling roles of FAK and FRNK in cardiomyocyte adhesion, growth, and survival.

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